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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 826-11 PCT/US

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INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CU99/00006	01 December 1999	02 December 1998

TITLE OF INVENTION

Preparations Containing Virus-Like Particles as Immunopotentiators Administered Through the Mucosa

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Copy of the international application as published with the International Search Report.

EXPRESS MAIL CERTIFICATE

Date 06/01/01 Label No. EL490332456US

I hereby certify that on the date indicated above, I deposited this paper or fee with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, D.C. 20231 by "EXPRESS MAIL Post Office to Addressee" Service.

CASSANDRA LEMS — Cassandra Lems

Name (Print) — Signature

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/857402	PCT/CU99/00006	976-11 PCT/US

24. The following fees are submitted:.	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	10 - 20 =	0	x $18.00	**$0.00**
Independent claims	1 - 3 =	0	x $80.00	**$0.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**
TOTAL OF ABOVE CALCULATIONS =				**$860.00**
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**
SUBTOTAL =				**$860.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	**$0.00**
TOTAL NATIONAL FEE =				**$860.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	**$0.00**
TOTAL FEES ENCLOSED =				**$860.00**
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a. ☒ A check in the amount of **$860.00** to cover the above fees is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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01 June 2001
DATE

PREPARATIONS CONTAINING VIRUS-LIKE PARTICLES AS IMMUNOPOTENTIATORS ADMINISTERED THROUGH THE MUCOSA.

Technical branch

The present invention is related to the branch of medicine, particularly with the use of new vaccine immunoenhancing strategies. In this case, the adjuvant is a virus-like particle (VLP), which at the same time constitutes an antigen of interest in the formulation. The adjuvant mechanism is based on the positive effect of one antigen on others or on the synergic interaction between the antigens of the formulation.

Previous technique

The technical objective pursued with the present invention is, precisely, the development of formulations capable of enhancing the immune response to antigens administered through mucosal routes, minimizing the number of components in the formulation. The enhancing activity is supported by the interaction between particles at the mucosal level, generating systemic as well as mucosal immunity. Furthermore, the development of combined vaccines to the mucosal route taking as a central antigen the HBsAg, increased the immune response to one or more of coadministered antigens. The obvious advantage is the elimination of all other element or compounds different from the antigen of interest and the use of a different route. We consider that this is the basis or nucleus to develop combined vaccines for a mucosal use.

HBcAg is an extremely immunogenic antigen during the Hepatitis B Virus (HBV) infection or after immunization. In many HBV chronic patients, this is the only antigen capable of inducing an immune response. It can even induce an immune response in mice in nanogram quantities. Recently, a few structural studies have demonstrated some important characteristics explaining its potent immunogenicity. HBcAg specifically binds membrane immunoglobulin receptors in a large number of resting B cells in mice, which is sufficient to induce costimulatory B7-1 and B7-2 molecules. In this way, non-sensitised B cells, specific for HBcAg can uptake, process and present HBcAg peptides to naive T cells *in vivo* and to T cells hybridoma *in vitro*, approximately

10^5 times more efficiently than macrophages and dendritic cells. This structure-function relationship explains the great immunogenicity of HBcAg (Milich, D.R. *et al.* 1997 Proc. Natl. Acad. Sci USA Dec23; 94(26): 14648-53).

Serologic and biochemical studies indicate that the resolution of HBV acute infection occurs in the context of an efficient cell-mediated immune response, while the chronic infection is characterized by a poor and undetectable cell-mediated immune response and a "relatively efficient" humoral response.

The humoral immunity and the cell-mediated immunity are regulated by different groups of helper T cells. Factors influencing the induction in mice of a Th1 or Th2 response to the HBV antigens (HBcAg/HBeAg) revealed that this balance was influenced (1) by the antigen structure (HBcAg is a particulated structure and HBeAg is not; (2) the major histocompatibility complex (MHC) of the host and the T cell antigens which are recognized; (3) the cross regulation between Th1 and Th2 cells; (4) the T cell tolerance, which is more complete for Th1 than for Th2 cells; (5) the activity of secreted HBeAg that preferentially delete Th1 cells (6) the treatment with cytokines, used to modulate *in vivo* the response toward Th1 or Th2 cells. This balance Th1/Th2 is relevant to the acute or chronic course of the HBV infection. Th2 cells preferentially evade the induction of tolerance compared with Th1. As HBeAg acts as a tolerogen during HBV vertical transmission, deleting Th1 cells, the predominance of Th2 specific cells for HBeAg could influence in the initiation and maintenance of a chronic carrier state. In this case the cytokine therapy endowed to modulate the response towards Th1, could be benefitious in the treatment of HBV chronic infection (Milich, D.R. 1997 J. Viral. Hepat.; 4 Suppl 2: 48-59).

The effect of HBeAg circulation on HBcAg Th1 specific T cells was examined by transferring HBeAg/HBcAg specific T cells to double (HBeAg and HBcAg) transgenic mice. The presence of serum HBeAg eliminated the Th1 mediated response against HBcAg and changed the balance to the Th2 phenotype. This result suggest that, in the context of the hepatitis B infection, circulating HBeAg has the potential to preferentially eliminate inflammatory specific Th1 cells needed for viral clearance, promoting the persistency of HBV (Milich-DR *et al.* 1998 J-Immunol. Feb 15; 160(4): 2013-21).

It is known that antibodies against HBcAg are present since the beginning of the infection and reach high concentrations in sera of HBV chronically infected patients, but these antibodies are not protective. Antibodies passively transmitted to newborn children by chronic carrier mothers, do not protect children of infection. (Beasley et al. 1977. American Journal of Epidemiology 105: 914-918). However, it has been demonstrated that immunizing chimps with HBcAg partially or completely protected them from HBV infection (Iwarson, S. *et al.* 1985 Gastroenterology 88: 763-767; Murray, K. *et al.* 1987 Journal of Medical Virology 23: 101-107). In Iwarson's study, three chimps were completely protected. After challenge with HBV, antibody levels against HBcAg and HBeAg increased but only one chimp seroconverted against HBsAg. In Murray's study, 2 out of 4 immunized chimps showed a low level of viral replication after challenge, HBsAg was detectable in sera for 2 or 3 weeks, and after that they developed an anti-HBsAg antibody response. It was hypothesized that the incomplete protection could be due to the low immune response in vaccinated animals without adjuvant.

After immunizing with woodchucks hepatitis core antigen (WHcAg) in Freund Complete Adjuvant (ACF), it was possible to protect woodchucks from challenge with the virus (WHV) without signs of infection detectable antibodies against the surface protein (WHsAg). Although the hypothesis that T helper anti-nucleocapsid immune response could enhance undetectable antibodies against the surface antigen can not be discarded, the cytotoxic activity was considered as the main responsible of protection (Roos, S. *et al.* 1989 J. Gen. Virol. 70, 2087-2095). In a second study using woodchucks the role of HBcAg and WHcAg in protection was determined as well as the possible mechanism. Animals were immunized with WHcAg and HBcAg and afterwards challenged using a high dose of WHV. In this experiment it was found that WHcAg is a protecting antigen there is a cross protection because 4 out of 6 woodchucks immunized with HBcAg were protected from the challenge. Both antigens generated a high antibody titer with a cross reactivity lower than 1%, confirming previous reports of protection using internal hepatitis B virus antigens. Since dominant B epitopes of both antigens do not appear to be

conserved, this result also demonstrated that antibodies directed against core antigens are not important for protection. Woodchucks immunized with WHcAg/HBcAg reacted with a rapid response of serum antibodies against surface proteins after challenge with WHV, indicating an increased helper T cell response as a potential mechanism of protection after immunization with an internal antigen of HBV/WHV. (Schodel-F *et al.* Vaccine. 1993; 11(6): 624-8)

Transfection of established cell lines from BALB/C mice hepatocytes with dimeric HBV DNA (ML lines) resulted in the expression of HBV antigens in these lines. The adoptive transference of spleen cells of BALB/c mice immunized with ML-1.1 cells expressing HBsAg as well as HBcAg, caused a regression of tumours cells expressing the corresponding antigens in athymic mice. Furthermore, the transfer of spleen cells of BALB/c mice immunized with HBsAg or HBcAg also caused tumoral regression. These results demonstrated that surface and nucleocapsid antigens could induce immunity capable of rejecting the hepatocellular carcinoma *in vivo* (Chen, S.H. *et al.* 1993 Cancer-Res. Oct 1; 53(19): 4648-51).

Therapeutic vaccines based on specific nucleocapsid epitopes for human HLA are being assayed in phase II/III studies (Liaw, Y.F. 1997 J.Gastroenterol. Hepatol. Oct, 12 (9-10): S227-35).

HBcAg has been demonstrated to be a very good carrier. HBcAg represents a highly immunogenic antigen in human and animal models. HBcAg activates directly B cells and generates strong T cell responses, furthermore, the efficient processing and presentation of HBcAg by the antigen presenting cells makes it the ideal carrier molecule. Hence a large number of epitopes has been chemically linked or genetically fused to the HBcAg molecule to successfully increase their immunogenicity. Expression vectors has been designed in bacteria to enable the insertion of heterologous B cell epitopes in different positions inside the particles of HBcAg and the efficient purification of hybrid particles.

Positional studies of B cell epitopes demonstrated that internal insertions by the amino acid 80 continue to be immunodominant, permitting an increase in the production of antibodies as compared to other fusion proteins.

Immunogenicity studies have been performed with experimental challenge in different systems. For example, a peptide from *Plasmodium berghei* Circunsporozoite was inserted in this site and the purified hybrid particle HBcAg/CS was highly immunogenic and protected 100% of challenged mice against malaria. Aimed to the development of oral vaccines, attenuated avirulent Salmonella strains have been used to introduce genes coding for hybrid particles of HBcAg (Milich, D.R. *et al.* 1995 Ann. N.Y. Acad. Sci. May 31; 754: 187-201).

In conclusion, apart from the relationship between HBcAg and protection, total or partially evidenced in chimps or indirectly referred by the experiments with WHcAg, this protein has a number of properties that makes it unique. HBcAg behaves as a T dependent as well as a T independent antigen (Milich, D.R. *et al.* 1986 Science 234, 1398-1401), it is very immunogenic, even without the help of adjuvants and its inoculation preferentially sensitises Th1 cells (Milich, D.R. et al. 1997, J. Virol. 71, 2192-2201). HBcAg is a very efficient carrier protein (Schödel, F. *et al.* 1992 J. Virol. 66: 106-114; Milich-DR *et al.* 1995 Ann-N-Y-Acad-Sci. May 31; 754: 187-20) and Th HBcAg specific cells mediate the antibody response against HBcAg as well as anti-HBsAg (Milich, D.R. et al. 1987 Nature (London) 329: 547-549). These immunologic characteristics are unique for the particulated HBcAg and do not apply to the non-particulated form of the antigen, the HBeAg (Milich, D.R. *et al.* 1997 Proc. Natl. Acad. Sci USA Dec 23; 94(26): 14648-53).

Detailed description of the invention

In the present invention it is reported for the first time a vaccine formulation having as main compounds: HBsAg and HBcAg in adequate proportions Other compounds may be introduced as stabilizers and preservatives.

The novelty of HBsAg/HBcAg formulation is linked to the anti HBsAg enhancing effect generated when HBsAg is mixed with HBcAg. Both antigens are compounds of HBV and hence, the role of the adjuvant is taken by other viral antigen attractive *per se* as a vaccine antigen, becoming a vaccine formulation with a wider anti-hepatitis B immune response spectrum. Other formulations of nucleocapsid antigens combined with surface antigens, for

example the formulation HBsAg/Virus like particle of Human Papilloma Virus and extended to other viral antigens, results in an increase in titers against both antigens. After mixing HBsAg with other antigens, an increase in the immunogenicity over other coinoculated antigens could be shown, evidencing a synergic effect produced by the combination X + HBsAg through the nasal route. In general, these results enables the generation of HBsAg mucosal combined vaccines, and enables the use of the positive interactions between VLP, considering VLP as organized proteic or lipoproteic structures, resembling viruses, with a size of nanometers.

In the case of the formulation containing HBsAg and HBcAg, we can obtain a superior product as compared to the single HBsAg commercially available vaccine because:

-It is possible to obtain a wider spectrum of immune response generated by HBcAg regarded as an important antigen *per se* in anti-VHB protection. Furthermore, the IgG seric levels anti-HBsAg reached by mucosal inoculation is as intense as the one obtained with the systemic inoculation in alum.

-The route of inoculation offers special advantages such as: systemic and mucosal immunity at the same time, the elimination of strong quality controls such as sterility and pyrogens as well as the high prices of injected vaccines, the related toxicity.

-The toxic effect generated by alum-based vaccines and the toxic effects of adjuvant injection can be avoided because the antigen number 2 is at the same time the adjuvant.

-It is possible to use the initial HBsAg + HBcAg formulation as a nucleus of combined vaccines.

-It is possible to immunize non-responders to the surface antigen and immunodepressed patients using this preparation, due to the inoculation route and the introduction of the nucleocapsid antigen regarded as a protective antigen *per se*.

-The characteristics of this formulation make it an ideal formulation for therapeutic use.

In the second place, nucleocapsid antigens, favour the increase of coinoculated antigens immunogenicity. We found a great simplicity of resulting formulations and, at the same time, the increased valence of these potential vaccines with a minimal number of antigens due to the possibility of avoiding the use of adjuvants, which are *per se* non-interesting antigens for protection. In this way very reduced combinations can be obtained if desired, for single or combined vaccines.

In the third place, it is possible to generate combined vaccines having as a nucleus the HBsAg whose immunoenhancing effect on other coinoculated antigens is demonstrated in the example 4. The advantages of these formulations are based on the effective association of HBsAg, as a central antigen of the anti HBV vaccine, with other antigens, with a demonstrated synergic effect in the generated response for both antigens. This fact, not only has the attraction of previously described variants but it also makes the HBsAg, -protecting antigen for a widely distributed world disease- the central antigen of combined formulations.

In general terms, compared to other mucosal vaccines, it is possible to detach the following advantages:

-The 'adjuvation' process –mixing antigens- does not require the adsorption of antigens, and the quantity of the HBcAg antigen is at similar levels of HBsAg.

-The filtration as a sterilizing process can be used due to the small size of the particles, while other strategies and adjuvants over 0.2μm can not be sterilized in this way.

-The simplicity of the production process for HBcAg makes it a very cheap antigen as compared to other adjuvants.

The formulations object of the present invention may present volumes from 0.01 until 10mL, depending of the inoculation route and the species to immunize. The antigen doses vary in a range of 0.001 to 1mg.

EXAMPLES OF PERFORMANCE

Example 1

With the aim of evaluating the immunogenicity of HBcAg through the nasal route, 3 groups of 8 female BALB/c mice were inoculated with a dose of 10μg

of HBcAg in all cases. The first group was inoculated with HBcAg in acemannan (CIGB, La Habana) 3 mg/mL (dry weight), adjuvant previously used to increase the immunogenicity of particulated systems through the nasal route. The second group was inoculated with HBcAg in phosphate-saline (PBS) buffer. Group 3 was injected subcutaneously with the antigen in alum and used as a control group for systemic inoculation. The schedule was followed of inoculations on days 0, 14, 28 and the extraction was done on day 42. The antibody response was quantified by immunoenzymatic assay (ELISA) to determine the IgG antibodies against HBcAg in sera. The Student's t test was performed to analyse statistically the results, $p<0.05$ was considered a significant difference.

It was demonstrated that, with the use of acemannan it was impossible to increase the anti-HBcAg antibody immune response. The antigen in PBS generated an immune response of a similar intensity to that obtained using acemannan (Fig. 1). The responses after nasal inoculation, in acemannan or in PBS, were similar to the response obtained using alum systemically. In conclusion, HBcAg can be used through the nasal route with a high immunogenicity.

Example 2

With the aim of demonstrating the immunoenhancing activity of HBcAg on HBsAg when both are mixed and inoculated through the nasal route, 4 groups of 8 female BALB/c mice were assayed. A two inoculations schedule was carried out. The inoculations were on days 0 and 14. The extraction was on day 21. The group 1 was inoculated with 10μg of HBsAg in PBS, group 2 with 10μg of HBsAg in acemannan (CIGB, La Habana) 3mg/mL (dry weight), group 3 with 10μg of HBsAg and 10μg of HBcAg. Group 4 was used as a systemic control, inoculating subcutaneously 10μg of HBsAg in alum (Fig. 2). The Student t test was performed to analyse statistically the results, $p<0.05$ was considered a significant difference.

From this experiment we concluded that it is possible to enhance the anti HBsAg immune response with the inoculation through the nasal route of HBsAg and HBcAg. The anti HBsAg response was significantly superior as

compared to the group when the HBsAg was inoculated in PBS and similar to that reached by the group inoculated in acemannan. The systemic inoculation of HBsAg in alum did not differ significantly from the groups inoculated with acemannan through the nasal route.

Example 3

With the aim of studying the enhancing effect of HBcAg at different doses in the murine model, 6 groups of 6 female BALB/c mice were selected. The schedule had three inoculations (days 0, 14 and 28) and two extractions (days 26 and 42). The assayed groups corresponded with: (1) HBsAg 5 µg in PBS; (2, 3 y 4) HBsAg 5 µg with 5, 10 y 20µg of HBcAg respectively, (5) HBsAg 5µg in acemannan 3mg/mL (dry weight) and (6) HBsAg 5µg in alum 0.5mg/mL. All groups except 6 were inoculated nasally. Group 6 was inoculated intramuscularly.

The Student's t test was used to analyse statistically the results, $p<0.05$ was considered a significant difference.

In this experiment we concluded again that it is possible to enhance the anti-HBsAg response with the coinoculation of HBsAg and HBcAg. The serum IgG response for the three immunized groups with both antigens was significantly higher to that obtained by inoculation of HBsAg in PBS and similar to that attained by the group inoculated in acemannan. We have demonstrated previously that acemannan increased the titers to levels similar to that obtained by cholera toxin in mice. Titers obtained by systemic inoculation in alum did not differ from that of acemannan and HBcAg/HBsAg groups by the nasal route. Although group 4 anti-HBsAg antibody response decreased as compared to group 3, the difference was due to a double increase in the HBcAg dose in-group 4. This increase might reduce the possibilities of HBsAg to penetrate mucosa.

Example 4

Different antigens were employed with the aim of studying the interaction of virus-like particles of Human Papilloma Virus 16 (VLP del VPH 16), HBsAg and HBcAg. Were immunized 8 groups of 6 female BALB/c mice with a

schedule based in inoculations on days 0, 14 and the extraction 7 days after second inoculation.

Comparing antibody titers against HBsAg, the response of acemannan formulation (group 6) has the same intensity to the HBcAg/HBsAg formulation (group 7) respectively. This is the third time that we demonstrate the enhancing effect of HBcAg.

From this experiment we also concluded that neither acemannan nor HBcAg enhanced antibody responses against VLP of Human Papilloma Virus (HPV), represented as groups 4, 5 and 8 in the third graphic. Statistical analysis using Student's t test ($p<0.05$ was considered a significant difference) did not show any difference between these groups.

Analysing the response against HBcAg in the group 5, where HBcAg and VLP of HPV were inoculated, low levels of antibody titers against HBcAg could be demonstrated as compared to group 7, where HBcAg was introduced along with HBsAg. Perhaps, the presence of these two particles antagonizes at the mucosal level. However, in group 2, high anti HBcAg and anti VLP of HPV could be achieved with the addition of HBsAg, being significantly higher the increase in these responses as compared to group 5 and do not differ from anti HBcAg response of group 7 (along with HBsAg). Hence we could realize a positive interaction between HBsAg and core antigens and a negative interaction between VLPs and HBcAg. The enhancing effect at mucosal level can occur in both senses, enabling the design of combined vaccines having as a nucleus HBsAg or the HBsAg/HBcAg combination.

HBsAg effect on group 2 not only enhanced the response against HBcAg, but it also enhanced the antibody response against the VLP of HPV. The same effect can be appreciated comparing the response against VLP between groups 1, 2 and 3 with group 8 where VLP were inoculated in PBS. Groups 1, 2 and 3 had statistically similar antibody levels, all of them higher than the group 8 level.

Group 1 (acemannan + HBsAg + VLPs HPV) and group 3 (HBsAg and VLP), did not differ in anti-HBsAg antibody titers. There was no statistical difference between group 3 and groups 6 and 7 (HBsAg/Acemannan and

HBsAg/HBcAg respectively). This result evidenced the enhancing effect of VLP of HPV on HBsAg immunogenicity.

These results support the use of combined formulations through nasal routes with HBsAg as a central immunoenhancing antigen. For example, the simple mixture of HBsAg and HPV VLP is very attractive and makes real the possibility of introducing more antigens, enhanced by the interaction with HBsAg.

The creation of complex formulations is possible without reduction of antibody response against each component, for example: VLP of HPV, HBcAg and HBsAg can be mixed without affecting IgG response against each component.

Example 5

With the aim of demonstrating the immunoenhancing activity of Hepatitis C Virus Nucleocapsid (HCV NC) on HBsAg when both are mixed and inoculated through the nasal route, 3 groups of 8 female BALB/c mice were assayed. A two inoculations schedule was carried out. The inoculations were on days 0, 14 and 28. The extraction was on day 42. The Group 1 was inoculated with 10μg of HCV NC, group 2 was inoculated with 5μg of HBsAg in PBS and group 3 with 5μg of HBsAg and 10μg of HCV NC in PBS (CIGB, La Habana) (Fig. 5).

The Student t test was performed to analyse statistically the results, $p<0.05$ was considered a significant difference.

From this experiment we concluded that it is possible to enhance the IgG response with the mucosal (IN) coadministration of HBsAg and HCV NC. The IgG serum response was significantly higher compared to the group immunized with HBsAg in PBS.

DESCRIPTION OF FIGURES

Figure 1. Three doses schedule (days 0, 14 and 28). Extraction was performed on day 42. Groups 1 and 2 were inoculated with 50μL through the nasal route. Group 3 was inoculated subcutaneously with 100μL.

Figure 2. Two doses schedule (days 0 and 14). Extraction was performed on day 21. Groups 1, 2 and 3 were inoculated with 50μL through the nasal route. Group 4 was inoculated subcutaneously with 100μL.

Figure 3. Three doses schedule (days 0, 14 and 28). Extraction was performed on day 26. Groups 1, 2, 3, 4 y 5 were inoculated through the nasal route. Group 6 was inoculated intramuscularly with 100μL.

Figure 4. Two doses schedule (days 0 and 14). Extraction was performed on day 26. All groups were inoculated nasally with 50μL. The composition of experimental groups is shown in table added to the figure.

Figure 5. Two doses schedule (days 0, 14 and 28). Extraction was performed on day 42. Groups 1, 2 and 3 were inoculated with 50μL through the nasal route.

CLAIMS

1. A vaccine formulation for nasal administration wherein the components are (a) a surface antigen from virus and (b) one or more vaccine antigens synergizing in adjuvant effect with (a), the antigen concentrations is in the range up to 1 mg each, preservatives and stabilizers can be added.
2. A vaccine formulation according to claim 1, wherein component (a) is the Hepatitis B Virus surface antigen and the component (b) is an antigen of viral nucleocapsid.
3. A vaccine formulation according to claim 1 and 2, wherein component (b) is a virus-like particle containing the nucleocapsid antigen of Hepatitis B Virus.
4. A vaccine formulation according to claim 1 and 2, wherein component (b) is a virus-like particle containing the nucleocapsid antigens of Human Papilloma-Virus.
5. A vaccine formulation according to claim 1 and 2, wherein component (b) is a virus-like particle containing the nucleocapsid antigens of Hepatitis C Virus.
6. A vaccine formulation according to claim 1, wherein component (a) is the Hepatitis B Virus surface antigen and the component (b) is a vaccine antigen of any nature or a mixture of different antigens receiving an immuno-enhancing effect from Hepatitis B surface antigen.
7. A vaccine formulation according to claims 1-6, administrated as a solid, liquid or spray product.
8. A vaccine formulation according to claims 1-6 for mucosal administration.
9. A vaccine formulation according to claims 1-6 for use as a therapeutic vaccine.
10. A vaccine formulation according to claims 1-6 for use as a preventive vaccine.

SUMMARY

The present invention is related to the branch of medicine, particularly to the new formulations of vaccine antigens.

The technical objective pursued with the present invention is, precisely, the development of formulations that are able to enhance the immune response to mucosally administered antigens, minimising the number of compounds in the formulation and generating strong mucosal and systemic responses through a synergic interaction between the antigens in the formulation.

These formulations enable: a) to broaden the spectrum of the anti-hepatitis B immune response, containing as main compounds HBsAg and HBcAg, b) to enhance the response against HBsAg with a viral nucleocapsid c) to generate combined vaccines through the mucosal route with HBsAg as a central antigen. Stabilizers and preservatives can be introduced.

The formulations of this invention can be applied in the pharmaceutical industry as human or veterinary vaccine formulations.

First schedule

1-10μg HBcAg / acemannan 3mg/mL	IN
2-10μg HBcAg / PBS 1X	IN
3-10μg HBcAg / alum 0.5mg/mL	SC

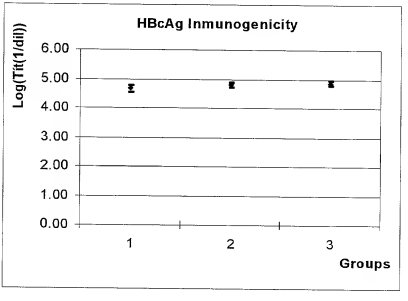


Fig. 1

Second Schedule

1-10μg HBsAg/ PBS 1X	IN
2-10μg HBsAg/ acemannan 3mg/mL	IN
3-10μg HBsAg/ 10μg HBcAg / PBS 1X	IN
4-10μg HBsAg/ Alum 0.5mg/mL	SC

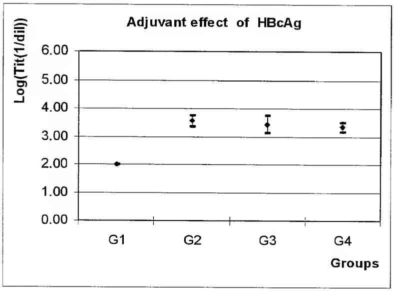


Fig. 2

Third schedule

1- 5µg HBsAg / PBS 1X	IN
2- 5µg HBsAg / 5µg HBcAg	IN
3- 5µg HBsAg / 10µg HBcAg	IN
4- 5µg HBsAg / 20µg HBcAg	IN
5- 5µg HBsAg / acemannan 3mg/mL	IN
6- 5µg HBsAg / alum 0.5mg/mL	IM

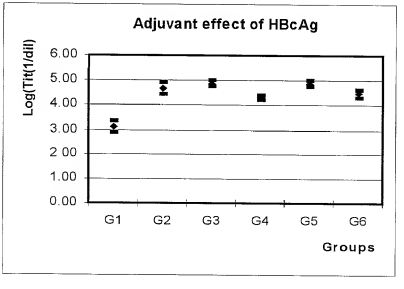
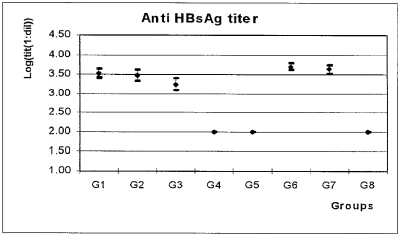
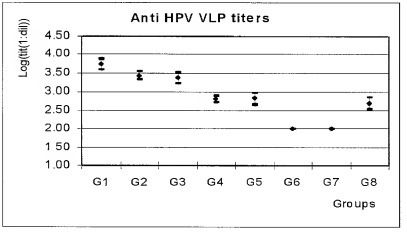


Fig. 3

Fourth Schedule: Synergism at mucosal level.

Acemannan 3mg/mL	X			X		X		
HBcAg 5µg/dose		X			X		X	
HBsAg 5µg/dose	X	X	X			X	X	
VLP /HPV 5µg/dose	X	X	X	X	X			X





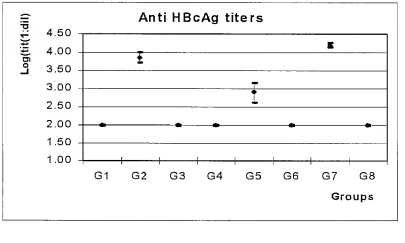


Fig. 4 Composition, per groups, in the upper part of the figure.

Fifth Schedule

1-10μg HCV NC/ PBS 1X	IN
2-5μg HBsAg/ PBS 1X	IN
3-10μg HBsAg/ 10μg HCV NC / PBS 1X	IN

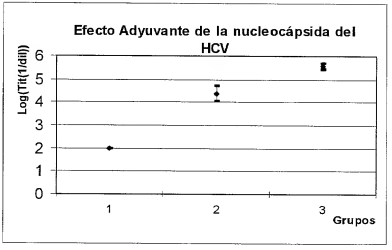


Fig. 5

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above, and that the filing of said specification, if heretofore filed, was authorized by me.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

CLAIM OF PRIORITY OF EARLIER FOREIGN APPLICATION(S) UNDER 35 U.S.C. §119(a)-(d)

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

(List prior foreign/PCT application(s) filed within 12 months (6 months for design) prior to this U.S. application.)

NOTE: Where item (c) is entered above and the International Application which designated the U.S. claimed priority check item (e), enter the details below and make the priority claim.

COUNTRY (or PCT)	APPLICATION NO.	DATE OF FILING *(Day/Month/Year)*	PRIORITY CLAIMED UNDER 35 USC §119
			[ ]YES [ ]NO
			[ ]YES [ ]NO

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S) UNDER 35 U.S.C. §119(e)

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

(List prior U.S. provisional applications)

PROVISIONAL APPLICATION NO.	FILING DATE *(Day/Month/Year)*

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S) UNDER 35 U.S.C. 120

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

(List prior U.S. applications or PCT international applications designating the U.S. for benefit under 35 U.S.C. §120.)

(b) [ ] was filed on_____ as
[ ] Serial No. _____or
[ ] Express Mail No. _____, as Serial No. not yet known
and was amended on _____. *(If applicable)*

(c) **[X]** was described and claimed in PCT International Application No. **PCT/CU99/00006**
filed on **01 December 1999** and as amended under PCT Article 19 on _____. *(If any)*

PATENT

Attorney's Docket No. 976-11 PCT/US

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION OR CIP)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type: *(check one)*

[X]Original
[ ] Supplemental
[ ] Design
[ ] National Stage PCT
[ ] Divisional
[ ] Continuation
[ ] Continuation-in-Part (CIP)

INVENTORSHIP IDENTIFICATION

NOTE: If the inventors are each not the inventors of all the claims an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted.

My residence, post office address and citizenship are as stated below next to my name.

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Daniel O. PALENZUELA GARDÓN	
Verena L. MUZIO GONZÁLEZ	
Gerardo E. GUILLÉN NIETO	
Eduardo PENTÓN ARIAS	
Dagmara PICHARDO DÍAZ	
Enrique IGLESIAS PEREZ	

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PREPARATIONS CONTAINING VIRUS-LIKE PARTICLES AS IMMUNOPOTENTIATORS ADMINISTERED THROUGH THE MUCOSA

the specification of which: *(complete (a), (b) or (c))*

(a) [ ] is attached hereto.

U.S. APPLICATIONS **STATUS** *(Check One)*

U.S. SERIAL NO.	U.S. FILING DATE *(Day/Month/Year)*	Patented	Pending	Abandoned
		[]	[]	[]
0 /		[]	[]	[]

PCT APPLICATIONS DESIGNATING THE U.S. **STATUS** *(Check One)*

PCT APPLN. NO.	PCT FILING DATE *(Day/Month/Year)*	U.S. SERIAL NOS. ASSIGNED (If any)	Patented	Pending	Abandoned
PCT/			[]	[]	[]
PCT/			[]	[]	[]

35 USC 119 PRIORITY CLAIM, IF ANY, FOR ABOVE LISTED U.S./PCT APPLICATIONS

PRIORITY APPLICATION NO.	PRIORITY COUNTRY	FILING DATE *(Day/Month/Year)*	ISSUE DATE *(Day/Month/Year)*

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith:

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DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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